Video Article

Key Elements of Photo Attraction Bioassay for Insect Studies or Monitoring Programs

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Abstract

Optimized visual attractants will increase insect trapping efficiency by using the target insect's innate behaviors (positive photo-taxis) as a means to lure the insect into a population control or monitoring trap. Light emitting diodes (LEDs) have created customizable lighting options with specific wavelengths (colors), intensities, and bandwidths, all of which can be customized to the target insects. Photo-attraction behavioral bioassays can use LEDs to optimize the attractive color(s) for an insect species down to specific life history stages or behaviors (mating, feeding, or seeking shelter). Researchers must then confirm the bioassay results in the field and understand the limited attractive distance of the visual attractants.

The cloverleaf bioassay arena is a flexible method to assess photo attraction while also assessing a range of natural insect behaviors such as escape and feeding responses. The arena can be used for terrestrial or aerial insect experiments, as well as diurnal, and nocturnal insects. Data collection techniques with the arena are videotaping, counting contact with the lights, or physically collecting the insects as they are attracted towards the lights. The assay accounts for insects that make no-choice and the arenas can be single (noncompetitive) color or multiple (competitive) colors. The cloverleaf design causes insects with strong thigmotaxis to return to the center of the arena where they can view all the options in a competitive LED tests. The cloverleaf arena presented here has been used with mosquitoes, bed bugs, Hessian fly, house flies, biting midges, red flour beetles, and psocids. Bioassays are used to develop accurate and effective insect traps to guide the development and optimization of insect traps used to monitor pest population fluctuations for disease vector risk assessments, the introduction of invasive species, and/or be used for population suppression.

Video Link

The video component of this article can be found at https://www.jove.com/video/57445/

Introduction

Almost all entomological surveillance depends on olfaction or visual attractants and often both. Volatile olfactory attractants may disperse throughout the environment resulting in a large attractive area. However, visual attractants may have a more limited range because of the invertebrate compound eye resolving images[1,2,3]. Therefore, visual attractants must be optimized to the insect of interest to maximize attraction and the trap designed to take advantage of the target insect's natural behaviors.

Visual attraction is based on wavelengths from the sun or other sources of light that are absorbed or reflected by an object's surface; organisms view this absorption/refraction of wavelengths as color. Insect vision has been found to include blue, green, and ultraviolet (UV) wavelengths[1]. Insects use their vision to aid in finding mates, food, and shelter[4]. Insects can visually define object sizes, colors, shapes, movements and contrasts[5,6]. Nocturnally active insects are generally attracted to light of differing contrast and intensity[4], whereas diurnal insects can resolve colors and images, in addition to contrast because of greater photon availability during the day. Monitoring traps use the insect's visual cues to their advantage to optimize attraction and capture[7].

The most common method of evaluating photo-attraction was observation of insect movement towards various colored shapes such as flowers[8] or objects (such as sticky cards[9,10]). Visual bioassays using colonized insects can help identify the optimal range of wavelengths and/or intensities, which reduces the number of field trials. Visual bioassays such as the "Two-Sided Light Tunnel" were designed for testing flies[11]. The problem with two sided light tunnels are that they do not account for insects that are not collected. Most insects will get stuck on internal corners and along edges in arenas. Also only two colors can be tested at one time. Other assays include the methods of Steverding & Troscianko (2004)[12], which narrowed tsetse fly attraction to broad bands (±50 nm) of light colors. Light emitting diodes (LEDs) have been incorporated into traps to improve insect attraction by optimizing the wavelengths of emitted light[1,13,14]. Optimizing the visual attraction of these traps or monitoring

devices will improve insect collection efficiency by using the insect's innate behaviors to lure insects. In this way, bioassay results are used to optimize existing trapping technology. The "Terrestrial Arthropod Trap" that improved the industry standard dome-type trap for red flour beetle surveillance (US patent# US8276314B2)) and the "Method and Compositions for Improved Light Traps" that incorporated of light emitting diodes into aerial insect traps (US patent# US2009/0025275A1). The two patents use LED technology that was optimized using the bioassay results to significantly improve insect traps.

This study describes a photo attraction bioassay arena and methods that allow investigators to evaluate the insect response to narrow wavelengths as a competitive or single attractive color. Equipment and experimental modifications are presented for nocturnal, diurnal, terrestrial, and aerial insects.

Protocol

1. Bioassay Components

1. **Terrestrial arena construction**
    1. Use metal flashing material strips of 2.54 cm length. Bend each strip into a half circle having a diameter of 15.24 cm (**Figure 1**).
    2. Connect the ends of each half circle and form a cloverleaf shape from the four pieces. Connect a locking nut and a #10 machine screw to the ends of each half circle to hold them together.
    3. Drill a hole at the midpoint of each semicircle 0.79 cm from the bottom of the arena. Affix 5 mm LED holders to the holes drilled in the middle of each semicircle.
    4. With the purpose of reducing reflection, spray matte black spray paint throughout the arena[14] to cover the metal flashing. Use a liquid texture (*e.g.*, Fluon) to prevent insects from climbing out of the arena over the spray paint.

2. **Aerial arena construction**
    NOTE: The aerial arena has a similar size and dimension to the terrestrial arena; however, polyacrylic material was used (**Figure 2**). The clear plastic allows all light to pass through. The clear plastic prevents reflection that interferes with the experiment. The clear plastic also enables the experiment to be filmed.
    1. Thread the furthest points of each semicircle to allow collection containers to screw onto the main arena. The cloverleaf shape sends insects back to the middle. No external corners encourage insect congregation; however, the collection cups for aerial insects have all internal corners and no external corners.
    2. For the aerial arena's collection containers, use screw top polymethylpentene containers (125 mL, 64 mm outer diameter, 74 mm height) and drill the bottoms (15 mm diameter).
    3. Into each of the containers' bottoms, affix threaded pipes (15 mm diameter, 60 mm length).
    4. Attach a 5 mm LED holder to the lids of each container. Thread each collection cage lid onto the large orifice of the collection cages.
    5. Seat the threaded pipe from the arena in the small orifice on the opposite side of the collection cage. Ensure that the entire threaded pipe end is flush with the walls inside the arena and fits tight to the collection cage.
        NOTE: The threaded pipe was made of Teflon. The Teflon glows the color of the LED that its collection cage holds. The threaded pipe was the only element that glows with respect to the insects in the arena due to a grey plastic substance at the base of each collection cage.

3. **Electronics preparation**
    NOTE: There are various colors (wavelengths) of LEDs are dependent on chemicals used to construct the LED and therefore a broad variety of colors are possible (**Table 1**).
    1. For all experiments, use standard 5 mm LEDs with positive and negative leads. The LEDs can be narrow in their range of wavelength ± 5 nm or can be large in their wavelength range ± 50 nm.
    2. Define the viewing angle as the maximum conical angle at which a display can be viewed. These are otherwise known as through hole LEDs. Through hole LEDs require either through hole slots on a PCB, a wiring harness, or wires soldered to the negative and positive terminals. Surface mount LEDs require proper PCB design and solder to incorporate them.
    3. Incorporate variable resistors into the electronics to control the LED power intake (LED intensity) (**Figure 3**). Use a light spectrometer to verify the intensity (W/m^2) and wavelength (nm) of the LEDs for each experiment.

2. Arena Preparation

1. **Before and between each replicate, carefully disassemble and clean the arena using an odorless, nonabrasive soap in warm water to remove any odors or unwanted attractants. Use a sponge with a low abrasive level to avoid scratching the arena.**
    1. Thoroughly dry the arena and set it aside to finish air drying in preparation for the next trial. This will prevent water spots from developing. Scratches and water spots can cause refraction on those points on the arena. Distortions create error in results.
    2. Whenever the arena must be handled, wear nitrile gloves to avoid introducing human odors onto the surfaces of the arena.

2. Record the following environmental conditions: humidity, temperature, barometric pressure, date, start/end time, external light sources, and LED positions in the arena. Record these values and monitor their trends from experiment to experiment. This ensures proper uniform experimental replicates, record the environmental conditions before and after the replicates.
3. **Types of experiments**
    NOTE: This setup is capable of single and competitive light testing.
    1. For single light testing, use one light emitting on a single cloverleaf while the rest of the clover leaves have nothing emitting from them.
    2. For competitive experiment, emit light from all four cloverleafs with different characteristics in competition with each other.

NOTE: Other experiments can assess the importance of insect state (fed, starved, teneral, mated, blood fed, *etc*.) and life history stage. Behavioral recording/analysis software can be used to record and quantify behavior. For nocturnal experiments, infrared cameras can be used to view the insects, which will glow white in the IR recording in contrast to the dark arena.

3. Rotate LED positions after every replicate to control the potential effect of light interference between opposing light sources and any environmental preferences.
4. To count the number of collections for insects who do not go into holes, use infrared LEDs, an infrared camera, and software[14]. The video recording will show the number of beetle visits to each LED. A collection is not counted unless the insect moves from the center of the arena toward an LED as opposed to following an edge past an LED.

4. **Arena setup**
    1. Set up a pedestal with four identical mason jars and place a black linen cloth on top of them. The linen cloth is black to keep light from reflecting off the bottom of the arena.
    2. Place the base plate of the arena on top of this pedestal. Assemble each piece of the arena on top of this base plate.
    3. Place the cloverleaf arena centrally around the release point in the base plate. Keeping this central allows the insects to emerge from the center of the experiment, giving them no initial preference.
    4. Install the light emitting diodes (LEDs) into the four collection container's LED holders.
    5. Set up the electrical equipment to control the lights.

3. Starting Bioassays

1. **Place the clear lid of the arena over the arena parallel to the baseplate. If insects are released through the baseplate, the arena lid should already be on the arena. This contains insects and allows visual assessment or video recording (terrestrial insects).**
    1. If necessary by species (aerial insects), temporarily immobilize the insects to allow extraction from their (emergence) cages and allow arena introduction. Knocking the insects down can be accomplished with temporarily with carbon dioxide or a cold temperature (< -20 °C for midges to -4.0 °C for mosquitoes).
    2. Using an aspirator, extract the desired sex and count of insects from the knocked down insects. Then, introduce the insects into the arena through the base plate. Use a pipe or other aspiration tool for insect extraction. Too much handling or long exposures will reduce survival.
    3. Start bioassay recordings/assessment before acclamation to confirm the insects are responding only to the light and not exhibiting an escape response. To avoid escape response, an provide an acclimation time of 1 h to the insects before powering on the electronics. Insects orient towards specific wavelengths of light during their escape response when placed into a new environment.

4. Ending and Quantifying Bioassays

NOTE: The duration of each experimental replicate will depend on insect behavior and response timing, in general use a longer exposure, more responses tend to be more informative.

1. Record environmental conditions.
2. Stop recordings such as the infrared camera, if used.
3. In the case of using collection chambers: after each replicate, place the cloverleaf arena into a freezer to kill the insects for quantification. The arena should not be left in the freezer for too long because the freezing environment may cause the plastic to crack.
4. Quantify insect behavior by counting insect responders in collection cages or analyzing video. Insects that remained in the cloverleaf arena were counted as having made no choice. For example, *Culicoides* were found to be most attracted to UV light compared with making no choice[7].

Representative Results

The terrestrial arena has been used to improve pest monitoring traps for red flour beetles[14] and the aerial arenas for hessian flies[15] and biting midges[7]. Although the cloverleaf arenas were similar, the conditions for each insect species were different and accommodated the evaluation of nocturnal or diurnal insects that can crawl or fly. More importantly these lab studies translated into field applications for monitoring insect pest population changes, introduction of invasive species, population suppression, and/or disease vector risk assessments.

The red flour beetles, a stored product pest, were evaluated in the terrestrial arena and filmed using an infrared camera[14]. Responses were considered positive for a color, if a beetle moved towards and contacted the LED. The arena setup was a competitive style with four lights or three lights and a dark blank for control. The trial data indicates the beetles were most attracted to near UV LED (390 nm) (**Figure 4**). This information was used to make a better red flour beetle trap using an octagonal UV LED array, which resulted in a 20% increase in collection compared to a 1% capture rate with the original pheromone attractant alone.

Hessian flies, wheat field crop pests were evaluated for photo attraction using the aerial arena with a diurnal setting[15]. Hessian flies were most attracted to green wavelengths with high intensities (**Figure 5**). Females preferred the green spectra of 502 and 525 nm. However, both sexes preferred high intensity light (16 W/m^2). This is the first report of Hessian fly attraction to select emitted wavelengths and intensities from LEDs under controlled conditions. These results are being used to develop a better Hessian fly detection trap for uninfested wheat fields.

The disease vector biting midge, *Culicoides sonorensis* can transmit viruses, which in cervids, ovids, and bovids may result in epizootic hemorrhagic disease or blue tongue disease. *C. sonorensis* were tested using the aerial arena under nocturnal conditions to determine the optimal colors that attracted sugar seeking biting midges[7]. The highest proportions of biting midges were attracted to ultraviolet (UV) light and light intensity was important with the brightest lights being most attractive (**Figure 6**). Sugar-seeking and escape behaviors were triggered by 355 nm and 365 nm in wavelength respectively and the biting midges distinguished between the two-colored lights. Using these wavelengths, the attraction of *C. sonorensis* to light traps can be improved and the lights have been incorporated into insecticidal sugar traps[16].

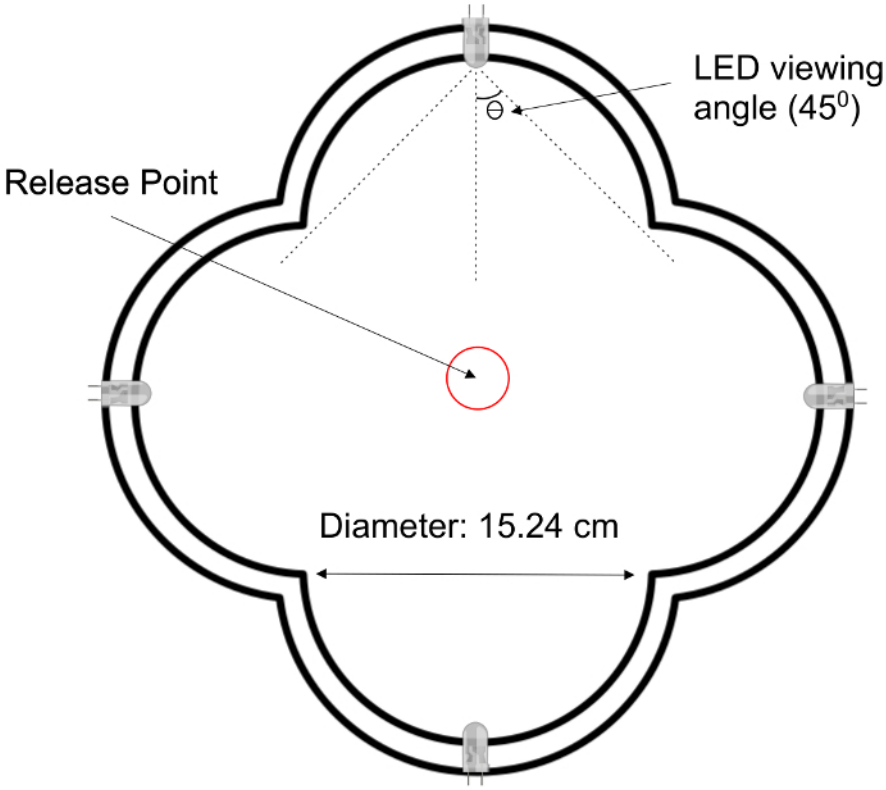


Figure 1: This drawing reflects the dimension of the Terrestrial arena. The release point at the middle of the arena as well as points of LED attachments at the apex of each half circle are labeled. Also presented is an example of a conical light projection from an LED. The optimal viewing angle of the LEDs is 45° although the arena design allows for more narrow or broad viewing angles as the half circles will limit light crossover except at the middle of the arena. The terrestrial arena has a lower profile compared to the aerial arena because the insects do not need space to fly, which helps video recordings stay focused on the insects. Please click here to view a larger version of this figure.

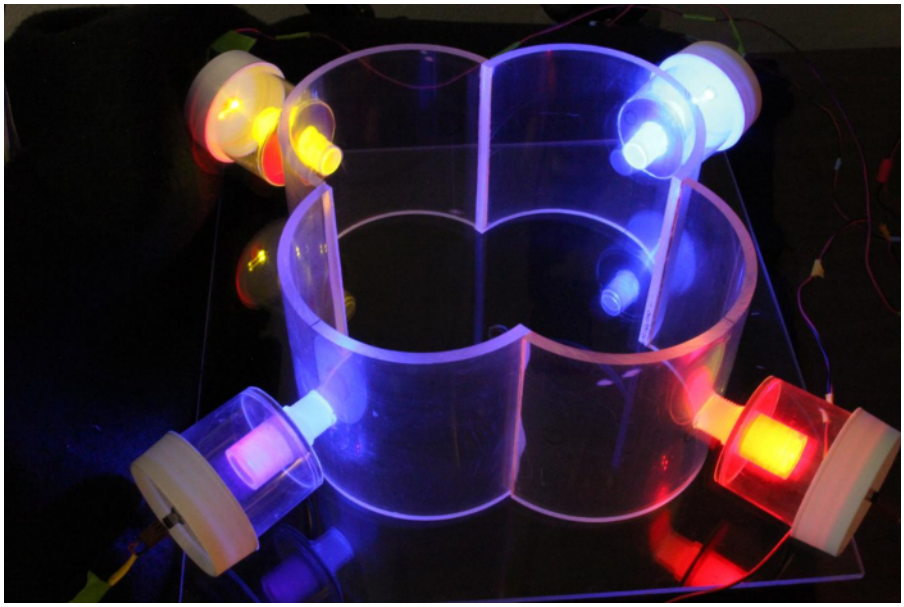


Figure 2: The aerial light assay arena constructed from clear acrylic although it has all the same design benefits of the terrestrial arena but allows for more vertical space for flying insect evaluation. Four collection containers have LEDs of various wavelengths illuminating their respective apex of the cloverleaf. This figure shows the arena set up competition style with red, green, blue, and UV lights. Please click here to view a larger version of this figure.

$R_{\#}$ – Potentiometer
V_S – DC Power Source
$D_{\#}$ – LED

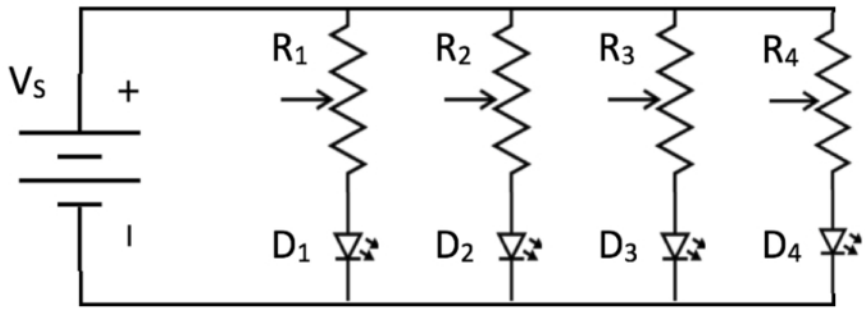


Figure 3: The electrical schematic of a 6 V DC power source attached to variable resistors (potentiometers) that control the power to each LED (light emitting diode) so the intensity of each LED can be adjusted independently. Neutral density paper can also be used to reduce the intensity without altering the emitted wavelengths. Wavelength and wavelength range are adjusted by selecting different LED chemistries. Please click here to view a larger version of this figure.

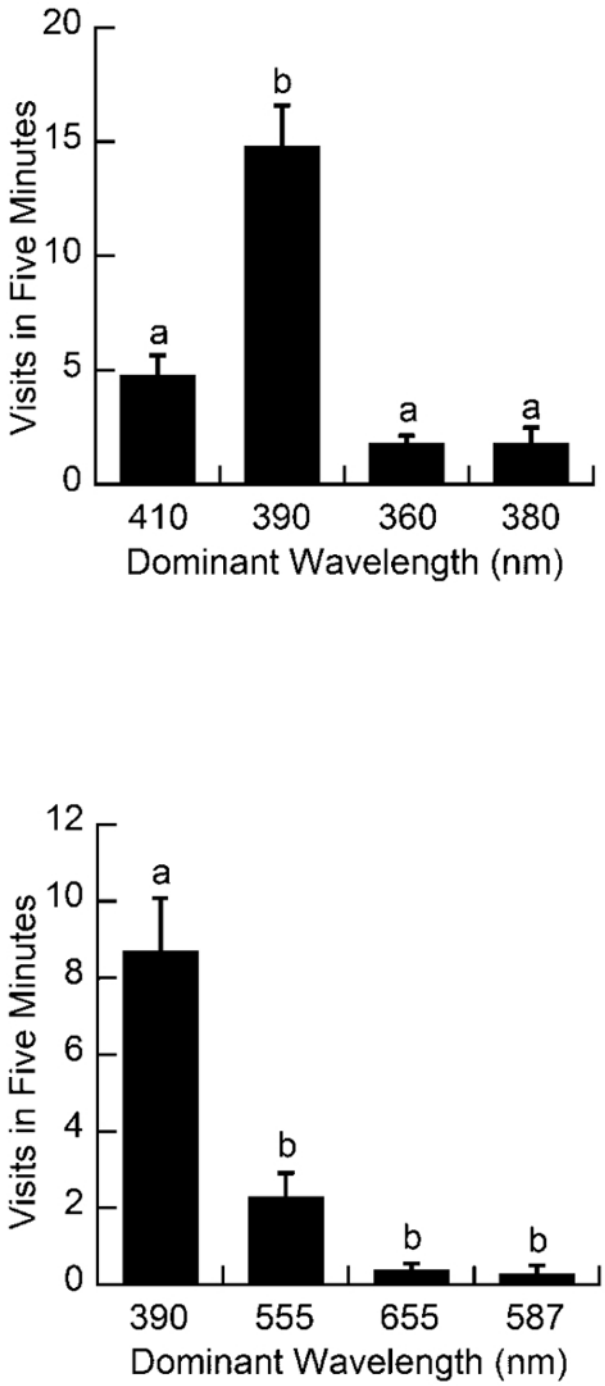


Figure 4: (Top) The movement of ten red flour beetles for 5 min was assessed in the cloverleaf arena. A visit was defined movement towards a color resulting in touching the LED. Tested colors were blue (410 nm) and UV (390, 380, and 360 nm). Standard error bars are indicated and significant differences are denoted by letters ($p<0.0001$), different letters indicate significantly different means. (Bottom) Further evaluation of movement with lower intensity colors was similar to above but with the colors UV (390 nm), green (555 nm), red (655 nm), and yellow (587 nm). (**Figure 4** was reprinted from Duehl *et al.* 2011 with permission.) Please click here to view a larger version of this figure.

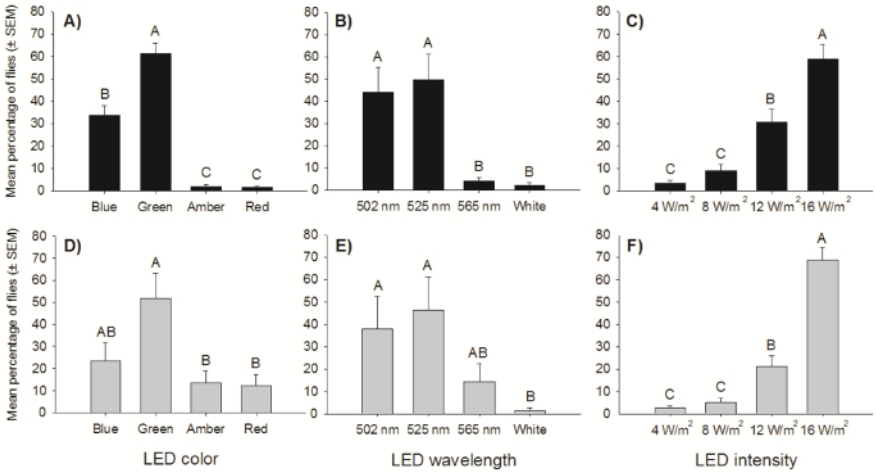
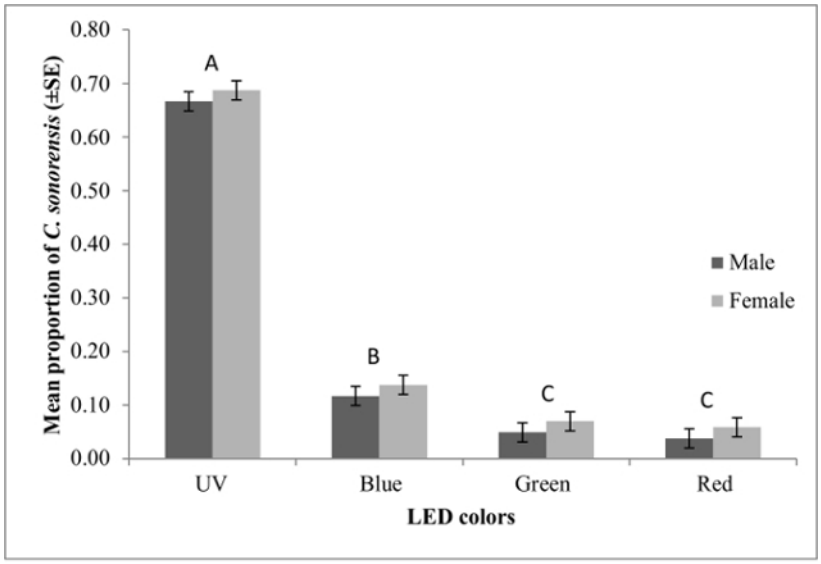


Figure 5: Males and female Hessian flies were evaluated for photo attraction separately to prevent cofounding factors. (**A-C**) are female fly responses and (**D-F**) are male. Significant differences are indicated by different letters ($P < 0.05$), different letters indicate significantly different means. (A and D) Both males and females were significantly attracted to green (527 nm) compared to red (624 nm), amber (590 nm), and blue (472 nm). (B and E) Within the green spectra 502-525 nm was most attractive and (C and F) intensity of light was important. (Figure was reprinted from Schmid *et al.* 2017 with permission.) Please click here to view a larger version of this figure.



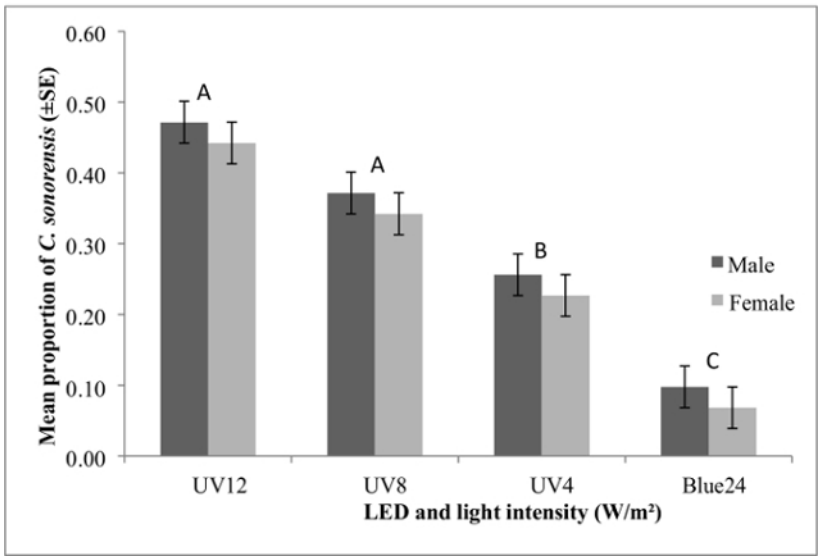


Figure 6: (Top) *Culicoides sonorensis* were attracted significantly more to UV light than blue, green, or red. Different letters indicate significantly different means (P <0.05), different letters indicate significantly different means. Sugar meals were provided prior to each replicate. (Bottom) Attraction to light intensity was assessed using *Culicoides sonorensis* movement towards the same UV light, but at different intensities (4, 8, and 12 watts) and a blue light (24 watts). (Figure was reprinted from Snyder *et al.* 2016 with permission.) Please click here to view a larger version of this figure.

Supplemental Table 1: General LED table for wavelengths. More narrow LED wavelengths do exist; this list just shows broad ranges of LEDs that exist in the insect's vision spectra. Please click here to download this file.

Discussion

Photo-attraction bioassays are an important tool to determine the optimal attractive color(s) and minimize the options for field trials of these colors. However, several factors must be considered when optimizing the bioassay for a specific insect including: Single Light *vs*. competitive light experiments, brightness, optimal spectral range, ambient light interference, state of the insects, and natural behaviors that may limit the possible responses.

Most insects have some phototaxis, which may be an innate escape mechanism causing the insect to move towards the light. This can be tested by providing a single light source in the arena and leaving the other three sides dark. However, a competitive test will have four colored lights and demonstrates color preference based on the insect response to each light. Bioassay users must determine if they are testing for light attraction or light preference. The competitive arena can be set up to look for repulsion as well. Remember that the insects may still not make a choice of light color, if they stay in the arena and do not orient towards a light. These no choice insects must be accounted for in the results.

Light emitting diode brightness must always be considered, and arena lights must be increased or decreased to the same intensity; therefore, it is important to test the brightness of the LEDs before each trial with a photo spectrometer. The potentiometers are important for controlling the voltage to each LED, which in turn adjusts the brightness. Commercially produced LEDs vary in voltage response and so even within a group of LEDs with the same spectra, each different LED must be evaluated, and the potentiometer adjusted before use. Even with this technique neutral density filters are sometimes needed to reduce the intensity of very bright bulbs. Snyder *et al.* (2016)[8] and Schmidt *et al.* (2017)[10] found brightness to be a significant factor in biting midge and Hessian fly collections with the brighter lights collecting proportionally more insects, although wavelength was the most important factor followed by brightness.

Bioassay users will benefit by testing narrow wavelength spectra LEDs. Snyder *et al.* (2016)[8] found *C. sonorensis* able to differentiate between wavelengths (10 nm apart) and these elicited very different behavioral responses. Narrow wavelength LEDs will therefore be necessary to determine the optimal narrow wavelength of light for a given behavior.

External light can interfere with light attraction. Schmidt *et al.* (2017)[10] found Hessian flies much more attracted to colors when given a dark arena than during a lighted one. However, in a crepuscular arena (partially lit), the lights worked the best. A dark arena blocks 100% of external light and is used to test nocturnal insects in their more natural visual environment. The arenas can also be used in natural light to simulate the visual environment of a diurnal insect, an important factor for ensuring attraction under real world trapping conditions.

Although visual attraction is important, olfactory attractants (pheromones, kairomones) can be added as in Duehl *et al.*(2010)[16]. This synergistic attraction increased trap collection. A long-distance attractant can aid in bringing individuals closer to the attractive light source and will greatly increase trap attraction[14]. For example, the pheromone used to attract the red flour beetles was a female sex pheromone. However, testing various stages such as fed, unmated, newly emerged, ovipositing, food/host seeking or other states may be important because they may have unique attractions as the may the various life history stages such as larvae, pupae, or adults. The trapping environment should also be considered, in food rich environments like flour mills food odor based attractants will be less efficacious.

Arenas may alter or influence insect behaviors even under controlled conditions such as fixed light levels, humidity, and temperature. The small areas or openings may be restrictive to natural insect movements. For example, in one trial *Culex tarsalis* mosquitoes did not enter the narrow openings in the collection cages (LW Cohnstaedt, personal observation) and house flies would not enter dark areas[11]. In some cases, these can be overcome by using sticky paper and catching the insects that go near the lights but will not enter cages or videotaping the insect behaviors. Therefore, all laboratory bioassay results need to be confirmed with field tests.

The light bioassay arena and protocol described are unique because they can be adapted to any terrestrial or aerial insect species. The arenas design accounts for high activity and low activity insects (the cloverleaf shape) and the lights are flexible for various competitive and non-competitive assays. Lastly this method can also accommodate most any life history trait (such as starved, sugar/host seeking, life history stage, *etc*.). These reasons help make this light bioassay a universal and flexible protocol for minimal time or money invested.

Disclosures

None

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